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Cellular and Nuclear Degradation during Apoptosis

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Abstract

Apoptosis ensures quick death and quiet clearance of unwanted or damaged cells, without inducing much, if any, immunological responses from the organism. In metazoan organisms, apoptotic cells are swiftly engulfed by other cells. The degradation of cellular content is initiated in apoptotic cells and completed within engulfing cells. In apoptotic cells, caspase-mediated proteolysis cleaves protein substrates into fragments; nuclear DNA is partially degraded into nucleosomal units; and autophagy potentially contributes to apoptotic-cell removal. In engulfing cells, specific signaling pathways promote the sequential fusion of intracellular vesicles with phagosomes and lead to the complete degradation of apoptotic cells in an acidic environment. Phagocytic receptors that initiate the engulfment of apoptotic cells play an additional and critical role in initiating phagosome maturation through activating these signaling pathways. Here we highlight recent discoveries made in invertebrate models and mammalian systems, focusing on the molecular mechanisms that regulate the efficient degradation of apoptotic cells.

Introduction

Among multiple types of cell deaths that have been identified, apoptosis stands out as a distinct type that is executed swiftly and quietly, without inducing much, if any, immunological responses in the organism. During an animal's life, a larger number of unwanted cells undergo apoptosis, a genetically programmed cell suicide process; these cells display several morphological changes including cellular shrinkage, chromatin condensation, nuclear fragmentation, and plasma membrane blebbing, yet retain their plasma membrane integrity and are rapidly internalized by other cells (Figure 1). The efficient demolition of apoptotic cells is a result of the degradation activities provided by both apoptotic cells and their phagocytes. Cell-autonomous degradation is initiated and executed by caspases, a family of *c*ysteine-dependent *asp*artate-directed prote*ases* that play determinant roles in apoptosis, and by caspase-activated proteases and nucleases [1]. After being swiftly engulfed by their neighboring cells or professional phagocytes through phagocytosis, an actin-based cell internalization process, apoptotic cells are sequestered in intracellular vacuoles referred to as "phagosomes" where they are degraded by a lysosome-mediated digestive activities (Figure 1) [2-4].

The efficient removal of apoptotic cells plays important roles in sculpting structures, maintaining homeostasis, and eliminating abnormal, non-functional, or harmful cells [5,6]. It is also an efficient tool for cell competition [7]. Moreover, this process prevents potentially

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harmful inflammatory and auto-immune responses that could occur if contents from apoptotic cells were leaked out [8]. Macrophages that engulf apoptotic cells even elicit anti-inflammatory responses that facilitate the resolution of regional inflammation [9-12]. Inefficient engulfment or degradation of apoptotic cells is associated with numerous chronicle inflammatory and auto-immune diseases [13-18]. In this review, we describe recent advances in our understanding of apoptotic cell degradation, focusing on four major topics: (1) caspase-mediated proteolysis and cell-autonomous degradation, (2) the multi-step degradation of nuclear DNA, (3) the role of autophagy in the removal of apoptotic cells, and (4) signaling pathways that regulate the maturation of phagosomes. This review does not cover many related topics such as the mechanisms that control the initiation of apoptosis, the exposure of "eat me" signals, the recognition and engulfment of apoptotic cells, cross-presentation of apoptotic cell antigens, and the fate of cells undergoing caspase-independent apoptosis, which are covered by other excellent reviews [4,19-21].

Caspase-mediated proteolysis initiates cell-autonomous degradation of apoptotic cell contents

The activation of initiator caspases by "intrinsic" or "extrinsic" apoptotic signals marks the beginning of apoptosis [22]. Initiator caspases further cleave and activate effector caspases, which subsequently process a large number of cellular substrates proteolytically [22]. These cleavage events are believed to lead to the signature cellular changes observed from apoptotic cells, which include cellular retraction, degradation of the nuclear envelope, chromatin condensation, degradation of nuclear DNA, and the release of signaling molecules that attract engulfing cells [1,23]. The initiation of nuclear DNA degradation by capsase-mediated activation of caspase-activated DNase CAD (also known as DFF40) is one of the best-studied examples (see the next section). In addition, caspase cleavage of nuclear lamins disassembles the lamin complex and weaken the nuclear envelope [24]. This event, together with the caspase cleavage and activation of rho-associated kinase I (Rock I) that further modifies the actin-cytoskeleton, have been proposed to result in the fragmentation of apoptotic nuclei [25]. The cleavage of Rock I plays an additional role in initiating membrane blebbing through cytoskeleton rearrangement [26-28]. A number of cytoskeletal proteins, including actins, tubulins, and actin-binding proteins, are caspase substrates [29-33]. Although the detailed mechanism have not been revealed, the degradation of cytoskeletal proteins is likely to contribute to cellular shrinkage during apoptosis.

An open web resource (The CASBAH: www.bioinf.gen.tcd.ie/casbah) was built in an effort to collect a comprehensive list of caspase substrates [31]. Despite the large number of potential caspase substrates reported, the list of caspase substrates that have been demonstrated to directly contribute to the degradation of apoptotic cells is surprisingly short. Previously, caspase substrates were mostly identified through *in vitro* cleavage assays, which were not able to distinguish whether a protein that was cleaved by a caspase *in vitro* was an actual caspase substrate during apoptosis. Recently, two research groups developed novel proteomic techniques, using which they identified global proteolytic events occurring during apoptosis [34,35]. Interestingly, the identities of the 261 and 333 proteolytic substrates reported by Dix et al. and Mahrus et al. [34,35], respectively, have only a minimum overlap with the caspase substrates previously documented in CASBAH, indicating that many more apoptotic-specific proteolysis substrates are yet to be found. The newly reported protein substrates implicate two surprising findings [34,35]. First, caspases do not cleave the entire proteome indiscriminately; rather, they often target multiple proteins within the same complex or biochemical pathways, suggesting a tendency for caspases to target specific pathways or signaling networks [35]. Secondly, many of the cleavage products are mapped to stably-folded functional domains, suggesting that rather than complete degradation of proteins, the apoptotic proteolytic cascades primarily generate new forms of proteins that may adapt new functions that further contribute

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to the self-killing event [34]. Future challenge resides at understanding the functional significance of the cleavage of the newly identified caspase targets *in vivo*.

The degradation of apoptotic nuclear DNA is a multi-step process

The degradation of nuclear DNA into oligonucleosomal fragments is a hallmark of apoptosis [36]. The massive cleavage of genetic materials irreversibly compromises DNA replication and gene transcription. Early in apoptosis, accompanied by chromatin condensation, chromosomal DNA is first cleaved into high molecular weight (HMW) fragments of 50-300 kb, which are subsequently processed into low molecular weight (LMW) fragments, the characteristic 180-bp DNA [37]. DNA fragments are readily detected *in situ* by the TUNEL (terminal deoxynucleotidyl transferase dUTP-mediated nick end labeling) assay, which labels 3′-OH end of DNA breaks [38]. After dying cells are engulfed by phagocytes, the partially digested DNA molecules are completely degraded into nucleotides in phagosomes [37]. A number of nucleases have been proposed to degrade apoptotic DNA, some of which act in apoptotic nuclei, whereas others in phagosomal lumen (Figure 2) [37,39-43].

CAD/DFF40 is the major cell-autonomous nuclease that accounts for most if not all activities for the generation of LMW DNA in apoptotic mammalian cells [44,45]. In cells in which CAD is deleted or inactive, inter-nucleosomal DNA fragmentation is either completely abolished or greatly reduced [18,46,47]. In living cells, CAD is in a complex with inhibitor of CAD (ICAD, as known as DFF45), which acts as CAD's folding chaperon during protein synthesis and subsequently inhibits its DNA cleavage activity [44,45,48,49]. During apoptosis, caspase 3 cleaves ICAD and releases CAD [44,45,50]. Initially, it was proposed that caspase cleavage of ICAD allowed CAD to enter the nucleus [44]. Subsequent evidence indicates that the endogenous ICAD/CAD complex resides in the nucleus of living cells; furthermore, the cleavage of ICAD that releases CAD appears to occur inside the nucleus [51,52]. The released CAD forms a scissors-like homodimer and cleaves double-strand DNA at nucleosomal linkers [53,54]. In addition, histone H1 might stimulate the enzymatic activity of CAD and also contribute to CAD's substrate specificity [55,56].

The residual DNA degradation activity detected in CAD-deficient cells suggests the existence of additional nuclease(s) during apoptosis [46,57]. Mammalian endonuclease G (endo G) is such a nuclease [57]. In living cell, Endo G resides in mitochondrial intermembrane space; upon apoptotic stimuli, it is released from mitochondria and translocated to nuclei, where it cleaves nucleic acids [57,58]. EndoGI, a recently identified EndoG inhibitor in *Drosophila*, is present in the nuclei of living cells and acts as a guardian for the accidental leakage of Endo G from mitochondria [59]. EndoGI is translocated to the cytoplasm upon apoptotic stimuli [59]. Additional CAD-independent DNases detected in apoptotic cells also include L-DNase II, apoptosis-enhancing nuclease (AEN), and DNase γ, which can be activated by different apoptotic stimuli [60-63].

The *C. elegans* genome does not encode any close sequence homolog of CAD. *C. elegans* NUC-1 (nuclease abnormal), a homolog of mammalian DNase II, is the first nuclease identified that drives the degradation of nuclear DNA in apoptotic cells [64,65]. In *nuc-1* mutant embryos, many apoptotic cells remain TUNEL-positive, whereas in wild-type embryos TUNEL-positive apoptotic cells are hardly detectable [65]. Genetic and cellular characterizations of *nuc-1* and *nuc-1*'s functional relationship with genes involved in the engulfment of apoptotic cells indicate the presence of at least three steps of apoptotic DNA degradation: the initial digestion that generates TUNEL-positive DNA ends, the conversion of TUNEL-positive to TUNEL-negative DNA-ends, which depends on NUC-1 activity, and the complete digestion of nuclei DNA into free nucleotides [65]. Although the expression pattern of NUC-1 has not been determined, genetic evidence suggests that NUC-1 is likely to act in apoptotic cells to mediate DNA

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degradation [65]. In addition, the *C. elegans* Endo G homolog CPS-6 and several other exo- and endonucleases form a DNA degradation complex named “degradosome” that acts in parallel to NUC-1 to promote DNA degradation [42,66,67].

Mice deficient in either CAD or Endo G are viable and develop normally [47,68-70]. Apoptotic events such as phosphatidylserine exposure, caspase activation and early-stage chromatin condensation are also normal in CAD deficient cells, indicating that the degradation of apoptotic DNA *per se* is largely dispensable for the initiation and execution of apoptosis [46]. Several reports, however, indicate active roles of cell-autonomous nucleases in the progression of apoptosis, especially in sensitive genetic backgrounds [67,71,72]. In addition, in certain cases the fragmented DNA was detected on the surface of apoptotic cells and was proposed to act as one type of the “eat me” signals that attract phagocytes [73,74].

The partially digested nucleosomal DNA is further degraded into nucleotides by other types of nucleases, primarily DNase II α, in the phagosomes of mammalian and *Drosophila* engulfing cells (Figure 2). DNase II α activity is optimal in acidic compartments such as lysosomes and phagolysomes [41,75]. In DNase II deficient flies and mice, a large number of undegraded DNA accumulated inside phagocytes [18,76,77]. The degradation of apoptotic-cell DNA plays active roles in preventing antigenic DNA from eliciting improper immune responses [78]. Undegraded apoptotic cell DNA in the macrophages of DNase II deficient mice induces an IFN regulatory factor 3/7-dependent production of IFNβ, which is cytotoxic and contributes to the lethal anemia in DNase II null mice [18,79-81]. Conditional knock-out of DNase II gene after birth causes adult mice to develop chronic polyarthritis that resembles human rheumatoid arthritis [82]. In mice that lack both CAD and DNase II activities, the undegraded DNA induces innate immunity and impairs thymic development [18]. Similarly, the innate immunity is induced by undegraded DNA in CAD(-/-)DNase II(-/-) flies [76]. In *C. elegans*, other than NUC-1, the DNase II homolog that likely acts in apoptotic cells, there must be additional functional counterparts of DNase II that act in phagosomal lumen to conduct cell-nonautonomous DNA degradation.

In summary, the nuclear DNA inside apoptotic cells is degraded in multiple steps by both cell autonomous and non-autonomous means. Cell-autonomous DNA degradation is dispensable for animal development since dying cells are subsequently engulfed by phagocytes and their DNA is effectively degraded by nucleases in phagosomes [68]. However, when massive apoptosis occurs and the degradation system is overloaded, the pre-cleavage by CAD may become essential [68]. Although the role of DNA degradation in apoptotic execution is largely elusive, the resulted DNA waste needs to be properly disposed to avoid the activation of innate immunity.

The contribution of autophagy to the clearance of apoptotic cells

Autophagy is a specific cellular event in which a portion of intracellular organelles and cytosolic components are engulfed by intracellular membranes and confined in a double-membrane vacuolar structure named autophagosomes, and are subsequently degraded by lysosomes that fuse with autophagosomes [83]. Autophagy is a stress adaptation process that generates energy and nutrients by degrading and macromolecules. Its relationship with apoptosis is complex. In many cases autophagy acts to save cells from the fate of apoptosis [84-88]; in other cases, when the swift apoptosis machinery is inhibited, starved cells or cells receiving death stimuli undergo an alternative form of cell death via autophagy [89,90]. In addition, during animal development, autophagic cell death has been observed to act as an independent form of programmed cell death [91-93]. Autophagy was also reported to potentiate caspase-dependent death [94]. The role of autophagy in the execution of cell death thus appears to be heavily dependent on the cellular and tissue context.

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The question most relevant to this review, namely, whether autophagy contributes to the cell-autonomous degradation of cellular contents during apoptosis, however, has not been answered. Interestingly, recently a new function of autophagy relevant to the ultimate degradation of apoptotic cells has been reported. In an *in vitro* system that mimics the cavitation of early mouse embryos, Qu et al. found that autophagy that occurred in apoptotic inner ectodermal cells contributed to the generation of ATP, which further promoted the exposure of phosphatidylserine, the “eat me” signal, on the surface of apoptotic cells, as well as the secretion of lysophosphatidylcholine, the “come-get-me” signal, to the neighborhood [95]. In this example, autophagy enables apoptotic cells to attract phagocytes, and thus indirectly facilitates their cell-nonautonomous degradation. A similar role played by autophagy has also been reported in chick retina [96]. On the other hand, ES cells in culture do not seem to rely on autophagy for the exposure of phosphatidylserine in response to apoptotic stimuli [95]. Whether the mechanism described above is commonly used by many kinds of apoptotic cells awaits further investigation.

Novel signaling pathways that control the maturation of phagosomes containing apoptotic cells

General knowledge about phagosome maturation

The maturation of phagosomes, a process that involves extensive remodeling of phagosomal membrane and contents and results in the eventual degradation of the engulfed particle, has been well characterized in mammalian phagocytes, such as macrophages, that ingest latex beads or opsonized microbes or red blood cells [97]. Once created, nascent phagosomes undergo sequential fusion events with intracellular organelles in the endocytic pathway, including early endosomes, late endosomes and lysosomes [97]. These fusion events promote the acidification of phagosomal lumen and deliver acid hydrolyses to phagosomes, which, in an acidic environment (pH<5.0), actively digest the protein, nucleic acid, and lipids confined in the phagosomal lumen [97]. A number of molecules, including phosphatidylinositol-3-phosphate (PI3P) and Class III PI 3 kinase Vps34, small Rab GTPases Rab5 and Rab7, V-type ATPase, and membrane fusion machinery components, were found to be recruited to phagosomal surfaces and drive phagosome maturation. The synthesis of PI3P on phagosomal surfaces, primarily conducted by Vps34, is believed to attract downstream effectors that are PI3P-binding proteins [98,99]. Rab5 and Rab7 act as membrane tethering factors for vesicles of different identities: Rab5 facilitates the early endosomes/phagosome fusion, whereas Rab7 facilitates the fusion of late endosomes and lysosomes to phagosomes [100-105]. V-type ATPase catalyzes the acidification of phagosomal lumen [106-109].

Special features of the maturation of phagosomes containing apoptotic cells

Until recently, little is know about how apoptotic cell are degraded inside phagosomes. Unlike macrophages that ingest bacteria, macrophages that engulf apoptotic cells secrete anti-inflammatory signals and actively suppress the secretion of the proinflammatory cytokines [9-12]. Further more, recent studies revealed that phagosomes containing apoptotic cells and opsonized-living cells matured at different rates [110]. These observations indicate the existence of mechanisms specific to the degradation of apoptotic cells. Recent research conducted in invertebrate model organisms and mammalian system revealed shared and unique mechanisms employed for the degradation of apoptotic cells.

The small nematode *C. elegans* has been a successful model for studying apoptotic cell death and apoptotic-cell engulfment [111,112]. Recently, owing to the establishment of multiple novel techniques, including the live-cell imaging in developing embryos and the genome-wide RNAi screen, and through the combined usage of these techniques with traditional genetic approaches, researchers have described in detail the different steps of the maturation process

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of phagosomes that contain apoptotic cells and identified a novel signaling pathway controlling phagosome maturation (Figure 3). It has been observed that the degradation of apoptotic cells in *C. elegans* also requires fusions of endosomes and lysosomes to phagosomes [113,114]. The recruitment of a series of key molecules, some of which previously unknown to be involved in phagosome maturation, to phagosomal surfaces drives these fusion events [113-117]. Below we summarize these new findings made in *C. elegans* as well as in other systems.

New executors of phagosome maturation that drive lysosomes/phagosome fusion

The Rab family small GTPases and their protein complexes are known to act as tethering factors that bring vesicles together for fusion [118]. Three *C. elegans* Rab GTPases, RAB-5, RAB-7, and RAB-2, have been found to play distinct roles during the degradation of apoptotic cells (Figure 3) [114-117]. Knocking out or down the activity of each of the three results in the accumulation of undegraded apoptotic cells. *C. elegans* RAB-7 is specifically required for the incorporation of lysosomes to phagosomes [114]. It mediates the extension of lipid tubules from phagosomes to recruit lysosomes, like mammalian Rab7 [105,114], and further promotes the fusion between these two compartments after docking of lysosomes on phagosomal surfaces [114]. In *rab-7*(RNAi) treated worms, phagosomes containing apoptotic germ cells are arrested as RAB-5-labeled phagosomes, suggesting that RAB-7 may act downstream or independent of RAB-5 [115]. The homotypic fusion and vacuole protein sorting (HOPS) complex is known to act as both an exchange factor and an effector for Rab7 during yeast endocytosis [119]. RNAi knockdown of each of all seven HOPS complex components causes persistent apoptotic cells in *C. elegans* gonads; further more, phagosomes are arrested at a RAB-7-positive stage, suggesting that the HOPS complex is likely to act downstream of RAB-7 [115]. In a separate study, Xiao et al independently discovered the function of the HOPS complex component VPS-18 in the degradation of engulfed apoptotic cells [120]. However, Xiao et al proposed that the major cause of the observed phagosome maturation defect is due to defects in lysosomal biogenesis caused by the *vps-18* mutations [120]. Whether and how the HOPS complex plays a direct role on phagosomal surfaces for lysosomes/phagosome fusion needs to be further investigated.

Unlike RAB-5 or -7, RAB-2 is a less-studied Rab GTPase whose function in phagosome maturation has not been revealed previously. *C. elegans* RAB-2 was identified from genetic screens for mutants that contain un-removed apoptotic cells [116,117]. Like RAB-7, RAB-2 plays an important role in the recruitment and fusion of lysosomes to phagosomes; however, unlike RAB-7, RAB-2 is also required for the acidification of phagosomal lumen [116]. RAB-2 and RAB-7 may control lysosome-phagosome fusions in parallel; alternatively, they may each contribute to a different subset of events. Proteomic studies in *Drosophila* and mammals have identified Rab2 as a component of phagosomes [121,122]. It remains to be elucidated whether mammalian or *Drosophila* Rab2 plays a conserved role in the maturation of phagosomes.

In addition to Rab GTPases, a novel function of the V0-ATPase in lysosomal/phagosomal fusion during the clearance of zebrafish apoptotic neurons has been identified through *in vivo* imaging [123]. This fusion activity is separate from the proton pump activity of the V-type ATPase [123], and is consistent with the membrane fusion activity reported for the fusion of yeast vacuoles [124]. In the near future, components of the membrane fusion machinery such as the SNARE complex and the regulators of this machinery are likely to show up on the list of novel apoptotic-cell degradation factors.

Key factors that regulate the phagosome maturation executors

Dynamins are conserved large GTPases that play pivotal roles in multiple membrane trafficking processes [125]. Dynamin's membrane fission activity underlines its essential function in driving endocytosis [125]. In other cellular context, dynamin and dynamin-related

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proteins are also known to promote membrane fusion [126-130]. In a genetic screen for mutants that are defective in both embryonic development and apoptotic-cell removal, fourteen loss-of-function alleles of *dyn-1*, the *C. elegans* dynamin gene, were identified [113]. Subsequent characterizations indicate that the function of DYN-1 is essential for both the engulfment and degradation of apoptotic cells [113,114]. DYN-1 drives the recruitment and fusion of early endosomes to phagocytic cups, an event that provides membrane material to support pseudopod extension around apoptotic cells [113]. Moreover, DYN-1 controls the recruitment and fusion of both endosomes and lysosomes to maturing phagosomes, a process critical for the delivery of multiple digestive enzymes and the V-type ATPase to phagosomes [113,114]. Specifically, DYN-1 acts as a mediator in a signaling pathway leading to phagosome maturation – it promotes the recruitment of RAB-7 to phagosomal surfaces and the synthesis of PI3P on phagosomal membranes [114]. DYN-1 thus acts as an upstream regulator of phagosome maturation effectors (Figure 3). In a genome-wide RNAi screen, Kinchen et al. also identified the function of *dyn-1* in phagosome maturation [115].

PI3P is generated on the phagosomal surfaces primarily owing to the activity of Class III PI-3 kinase Vps34 and functions there to recruit downstream factors, such as proteins with Phox (PX) or Fab1-YOTB-Vac1-EEA1 (FYVE) domains [97]. In *C. elegans* engulfing cells, PI3P is synthesized on nascent phagosome surfaces immediately after the internalization of apoptotic cells and remains present throughout phagosome maturation [114]. RNAi-mediated inactivation of *C. elegans vps-34* results in a mild increase in the number of apoptotic germ cells and *vps-34* was proposed to function under the control of DYN-1 to synthesis PI3P [115]. Given that *vps-34* RNAi, unlike *dyn-1* mutations or RNAi, only causes mild apoptotic-cell retention phenotype, there might be additional PI 3 kinases that act in parallel to generate phagosome-specific PI3P in response to DYN-1.

The role of Rab5 GTPase appears to be more complex. During the maturation of phagosomes containing microbes or opsonized particles, Rab5 is proposed to act as a tethering factor between early endosomes and phagosomes [102,131,132]. In both *C. elegans* and mammalian cells, recent studies found that Rab5 also promotes the maturation of phagosomes containing apoptotic cells [115,133]. Since the incorporation of early endosomes to phagosomes is a crucial step during the degradation of apoptotic cells [113], it is likely, although proof is still needed, that the tethering factor function of Rab5 is conserved during apoptotic-cell degradation. Besides this executor function, Rab5 also regulates downstream signaling events. In the endocytic pathway, Rab5 was known to activate Vps34 and promote PI3P synthesis on the target membranes [134-136]. Recently, Kinchen et al propose a different model in which Vps34 activates Rab5 by mediating the interaction between Rab5 and dynamin 2, based on protein-protein interaction studies in mammalian cells and genetic studies in *C. elegans* [115]. Whether this model can be reconciled with the observations made in the endocytic pathway and the model proposed by Kitano et al [133] in Rab5 activation requires further investigation.

Kitano et al observed that the activation of Rab5 on the surface of nascent phagosomes containing apoptotic cells is dependent on EB1, a microtubule-tip-associating protein that also interacts with Gapex-5, a guanine nucleotide exchange factor (GEF) for Rab5 [133]. Kitano et al thus propose that the recruitment of Gapex-5 to phagosomes through the microtubule network leads to the subsequent recruitment and activation of Rab5 [133]. The identification of Gapex-5 and EB1 as essential factors provides a molecular mechanism that involves the novel and critical role of microtubules for the regulation of Rab5.

Phagocytic receptors acting as the initiators of phagosome maturation

As an essential regulator of phagosome maturation that controls the recruitment and activity of multiple downstream regulators and executors, how is DYN-1 regulated? First of all, the

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association of DYN-1 to extending pseudopods and nascent phagosomes is critical for its function in the removal of apoptotic cells [113]. Furthermore, the recruitment of DYN-1 to pseudopods and nascent phagosomes is dependent on the phagocytic receptor CED-1 and its adaptor protein CED-6 [113]. Lack of DYN-1 enrichment to the surfaces of pseudopods and nascent phagosomes, as a consequence of *ced-1* or *ced-6* mutations, causes severe defects in engulfment and degradation of cell corpses [113,114]. Consistent with this mechanism, epistasis analysis places *dyn-1* in the signaling pathway composed of *ced-1* and *ced-6* [113]. These results indicate that vesicle trafficking is a novel event regulated by the CED-1 pathway; they further imply that CED-1, by controlling DYN-1 activity, also regulates phagosome maturation [113,114].

CED-1 and CED-6 are members of one of the two previously identified *C. elegans* signaling pathways that are believed to specifically control the engulfment of apoptotic cells [137-139]. The novel functions of CED-1 and CED-6 in phagosome maturation were overlooked previously because strategies that distinguish engulfed vs. unengulfed apoptotic cells in real time were not established [114]. With the aid of the newly developed live-cell imaging technique, Yu et al discovered that like *dyn-1* mutations, *ced-1* mutations not only greatly reduce the efficiency of engulfment but also impair the degradation of those apoptotic cells that are engulfed inside phagosomes [114]. Signaling events that require CED-1 activity, including the recruitment of DYN-1 and RAB-7 to and the synthesis of PI3P on the surface of phagosomes, also require CED-6 [114]. As a consequence, *ced-1* and *ced-6* mutants are both defective in the recruitment and fusion of early endosomes and lysosomes to phagosomes [113,114]. Although CED-1 is only transiently localized to phagosomal surfaces, it co-exists with DYN-1 for a period of time [113,114]. Thus, through CED-6, CED-1 recruits DYN-1 to phagosomes, which promotes a downstream signaling cascade that leads to apoptotic cell degradation (Figure 3) [113,114].

Previously, phagocytic receptors were only known to recognize phagocytic targets and initiate their engulfment. The above finding reveals that in addition to this well-known function, CED-1 plays a novel role in phagosome maturation. It further indicates that phagosome maturation is not a process that occurs spontaneously once a phagosome forms, rather, signaling from the phagocytic receptor is needed to initiate this process. Moreover, this finding suggests that different phagocytic receptors may promote different phagosome maturation modes and subsequently induce phagocytes to elicit different responses, including different immune responses. CED-1 belongs to a family of transmembrane proteins whose extracellular domains are of large sizes and contain an N-terminal emilin (EMI)-like domain followed by tandem repeats of an atypical EGF like repeat motif [140]. Draper, the *Drosophila* ortholog of CED-1, like CED-1, is known to be essential for the engulfment of apoptotic cells as well as pruned axon fragments [141-144]. Interestingly, Kurant et al [145] recently observed that in *draper* mutants, apoptotic cells are retained inside phagosomes for a prolonged period of time. Based on their genetic and cell biological characterizations of single mutants of *draper* and *simu*, which encodes another EMI-domain and EGF-repeats containing transmembrane protein, and of *draper*; *simu* double mutants, Kurant et al. further propose that SIMU is primarily involved in the recognition and uptake of apoptotic cells whereas Draper is primarily required for the degradation of apoptotic cells [145]. These findings indicate a conserved role of the CED-1 family of phagocytic receptors in phagosome degradation in worms and flies. It remains to be elucidated whether mammalian homologs of CED-1, such as human mEGF10 [138,146], and other phagocytic receptors for apoptotic cells also provide the initiation signal for phagosome maturation in addition to promoting the engulfment of apoptotic cells, and furthermore, whether the initiation of phagosome maturation is a common function performed by all phagocytic receptors.

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Cytoskeleton reorganization might also play a role in the degradation of apoptotic cells

CED-5, the *C. elegans* homolog of mammalian protein Dock180, is a component of a bipartite nuclear exchange factor for CED-1/Rac1 GTPase, and acts in a signaling pathway together with CED-10 but in parallel to CED-1 to promote the engulfment of apoptotic cells [112]. Recently, it was observed that CED-5 acted in a distinct pathway to control phagolysosome formation during the degradation of apoptotic cells [114]. During engulfment, the pathway led by CED-5 was known to regulate cytoskeletal reorganization [112]. Cytoskeletal reorganization also plays an active role in phagosome maturation in mammalian cells [110, 147]. CED-5 and other members of its pathway thus might contribute to phagosome maturation through remodeling the cytoskeleton.

Concluding remarks

Studies focusing on the degradation of apoptotic cells provide a wonderful platform for investigating a number of fundamental biological processes, including, but not limited to, how apoptotic execution machinery coordinates the multiple cellular demolishing events, whether and how autophagy, another fundamental cellular activity, is involved in the clearance of apoptotic cells, how the initiation and completion of apoptotic cell degradation in two different cell types are coordinated, and the identity of the components and organization of the signaling pathway(s) for recruiting intracellular vesicles to support phagosome maturation. The usage of model organisms further places the clearance of apoptotic cells in a whole animal context. However, what we know currently, as summarized in this review, is only the tip of an iceberg. Without repeating the questions for future exploration that have already been spelled out in the text, here I would like to list several interesting questions that have not been well explored. First, what is the exact role of cell-autonomous degradation of apoptotic cells? It seems that the caspase-initiated DNA degradation is dispensable under physiological conditions since DNA degradation occurring inside phagosomes provides a backup activity. However, other events, such as the exposure of “eat me” signals, cell retraction and detachment from the surrounding tissue, which are essential for ensuring that apoptotic cells are to be engulfed by phagocytes, may rely on caspase-mediated cleavage of multiple substrates. Exploring this aspect will lead us to further understand the relationship between apoptotic cells and there neighbors. Secondly, autophagy was associated with phagosome maturation in recent studies [148,149]. A comprehensive study of the contribution of autophagy in both apoptotic cells and phagocytes to the degradation of apoptotic cells will shed light on the relationship between autophagy and apoptosis. Last but not least, the cell-non-autonomous degradation of apoptotic cells has established a new model for studying the mechanism of phagosome maturation. The finding that phagocytic receptors for apoptotic cells are crucial in initiating phagosome maturation provides a new clue to understand the distinct immune responses phagocytes generated against different phagocytic targets. The detailed molecular mechanisms behind each step of phagosome maturation, such as how dynamin serves as a mediator of phagosome maturation, what the PI3P effectors are, and the functional relationship among Rab GTPases 2, 5, and 7 all await to be explored in worms, flies and mammals.

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one of the down stream effectors, RAB-7 localizes on phagosome surface shortly after engulfment to mediate the fusion of lysosome to phagosome and its function is crucial for degradation of apoptotic cells.

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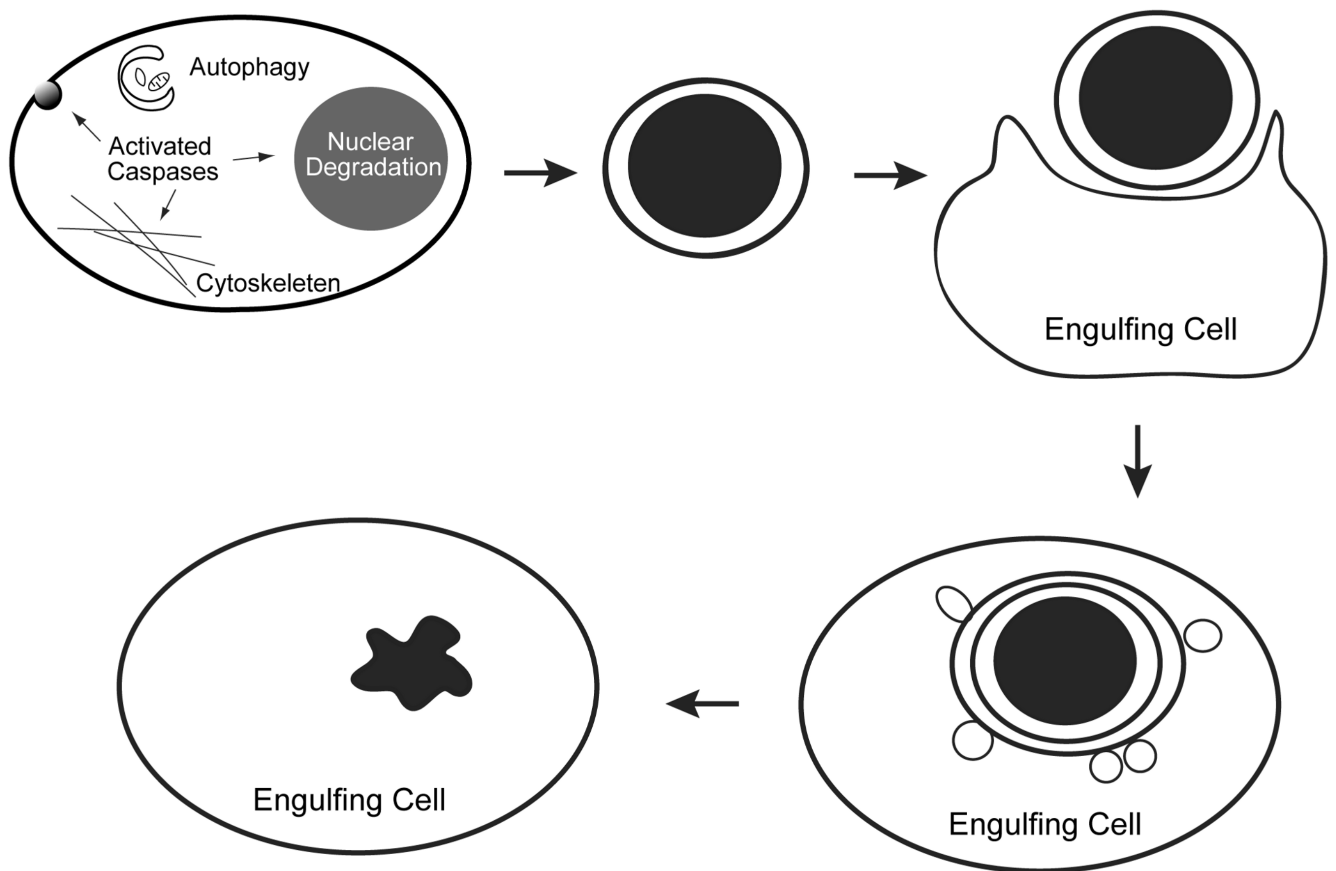

Figure 1.

The process of apoptotic cell removal including cell autonomous and cell nonautonomous degradations. (i) When cells adapt the apoptotic death fate, activated caspase family proteolyses are responsible for the degradation of cytoskeleton, releasing and exposure of signals that attract phagocytes and induce DNA fragmentation by activating DNases. (ii) Apoptotic cells are recognized and engulfed by phagocytes. (iii) Inside the phagocytes, apoptotic cell containing phagosomes fuse with different intracellular organelle species. This fusion process dramatically changes the membrane and lumen component of the phagosome and facilitates the complete degradation of apoptotic cells.

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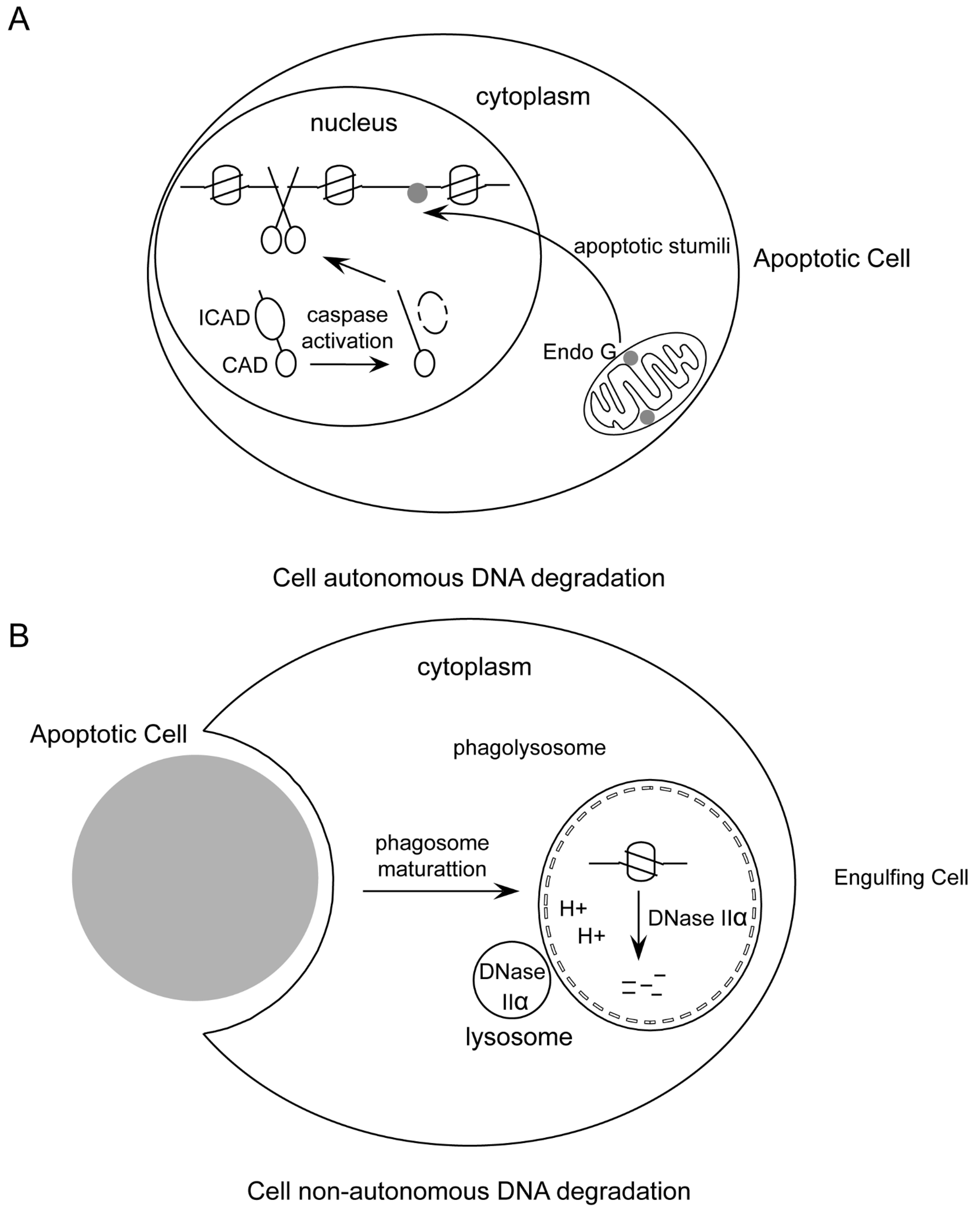

Figure 2.

In living cells, the activity of CAD is inhibited by ICAD and the Endo G is sequestered in mitochondrial intermembrane space. During apoptosis, the activated caspases cleave ICAD and release CAD, which forms homodimer and cleave linker DNA between necleosomes. The activation of caspases also triggers the release of Endo G from mitochondria into nucleus to cleave chromosomal DNA. B. After engulfed by phagocytes, the apoptotic cell resides in phagosome. Through phagosomal maturation, the phagosome acquires different digestive enzymes including DNase II α from lysosomes and its lumen is gradually acidified. In acidic condition, the active DNase II α further degrades nucleosomal DNA into nucleotides.

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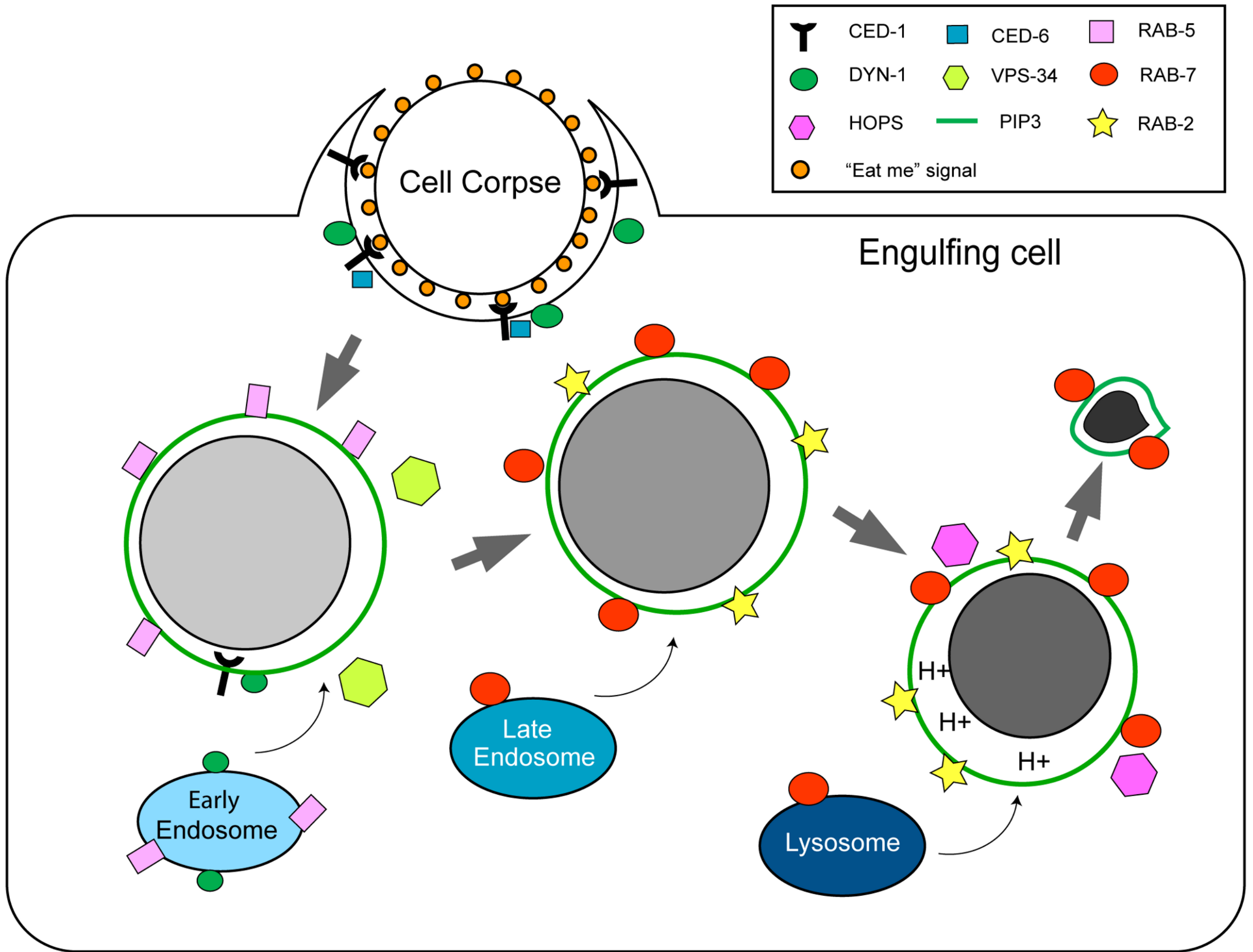

Figure 3.

Cell non-autonomous degradation of apoptotic cells. In *C. elegans*, the signal cascade of apoptotic cell degradation start from phagocytic receptor CED-1 and followed by CED-6, large GTPase DYN-1, small GTPases (RAB-5, RAB-7 and RAB-2), class III PI3 kinase Vps-34 and members of HOPS complex. DYN-1 and RAB GTPase localize on phagocytic cup or phagosome surface to regulate the sequential fusion of intracellular organelles, including early and late endosomes and lysosomes, to phagosome. PIP3 are syntheses on the phagosome surface mainly by Vps-34 and serve to recruit downstream effectors. Members of HOPS complex are RAB-7 effectors and function mainly downstream of RAB-7. It is not know whether Vps-34 and HOPS complex also localize on phagosome surface. During phagosome maturation, its lumen pH level drop from near neural to below 5, which activate the capthesin family proteases and DNase II to complete degrade phagosome contents.